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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/87 A1

(11) International Publication Number: WO 96/14424

(43) International Publication Date: 17 May 1996 (17.05.96)

(21) International Application Number: PCT/GB95/02612

(22) International Filing Date: 8 November 1995 (08.11.95)

(30) Priority Data: 9422495.3 8 November 1994 (08.11.94) GB

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(81) Designated States: AL, AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TT, UA, UG, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, LS, MW, SD, SZ, UG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DNA TRANSFER METHOD

(57) Abstract

A method for transforming a cell with a nucleic acid comprising contacting the cell with a vector which comprises the nucleic acidin the presence of a protein having a high basic amino acid content.

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DNA Transfer Method

The present invention relates to an improved method of transferring DNA into cells, particularly by transfection. In particular, the invention concerns the use of proteins having a high basic amino acid content in order to improve efficiency of DNA transfer and the use of calcium nitrate in a calcium phosphate transfection protocol.

The transfer of cloned DNA into mammalian cells is a routine procedure widely used in a number of applications, including basic research into the mechanisms of action of cellular machinery, protein expression using recombinant DNA techniques, the creation of transgenic animals and gene therapy. A variety of different techniques are available for the transfer of cloned DNA. These techniques include the use of viral vectors, direct injection into the cell and transfection in which the DNA is taken up directly by the cell. A number of different transfection techniques exist, such as DEAE-dextran mediated transfection (McCutchan and Pagano, 1968) and calcium phosphate mediated transfection (Graham and van der Eb 1973). A number of other related procedures include electroporation (Potter *et al*, 1984), liposome technology (Schaffer-Ridder *et al*, 1982) and lipofection (Felgner et *al*, 1987).

Still the most common technique is calcium phosphate mediated transfection. This technique involves mixing DNA directly with calcium chloride in a phosphate buffer. A calcium phosphate precipitate containing the DNA forms and this precipitate adheres to the surface of the cells to be transfected. The precipitate, including the DNA, is then taken up into the cell by endocytosis.

We have now found that proteins rich in basic amino acids may be used to dramatically increase the efficiency of transfection processes. According to a first aspect of the present invention, therefore, there is provided a method

for transfecting a cell with a nucleic acid comprising contracting the cell with a vector which comprises the nucleic acid in the presence of a protein having a high basic amino acid content.

The nucleic acid used to transform the cells may be in the form of DNA or RNA and may encode any protein or ribonucleic acid of interest.

The vector may be any vector used for transfection, such as a plasmid, in circular or linearised form.

Preferably, the vector is delivered to the cell using a transfection process known to those of skill in the art. Preferably, the transfection process is calcium phosphate mediated transfection. However, it is envisaged that other processes which involve the adherence of DNA to the cell surface will be enhanced by the use of the improvement of the invention.

The basic amino acid rich protein is preferably a histone protein. Advantageously, the histone protein is histone H2A.

In the case of calcium phosphate transfection, the protein is advantageously added to the transfection mixture after the formation of the calcium phosphate precipitate. However, satisfactory results may be obtained even if the histone is present *ab initio*.

A further improvement in transfection efficiency may be achieved by replacing the calcium chloride in the transfection protocol with calcium nitrate. Use of calcium nitrate is found to give a measurable improvement in transfection efficiency even when used independently of histone proteins. However, when used in conjunction with histones a synergistic effect is observed which leads to a large scale increase in transfection efficiency, sometimes

over 400 fold.

The invention further provides a kit for putting the method according to the previous aspects of the invention into practice. Preferably, the kit comprises at least one of:

a) a preparation containing a protein having a high basic amino acid content;

b) calcium chloride and/or calcium nitrate;

c) a phosphate buffer; and

d) nucleic acid.

The invention is described below for the purposes of exemplification only, with reference to the following figures, in which:

Figure 1 shows the transfection of neuroblastoma N2A cells by the calcium phosphate method, using varying amounts of histone H2A;

Figure 2 shows transfection of 3T3 fibroblasts by the calcium phosphate method using varying amounts of histone H2A.

1. Effect of Histone with the Calcium Phosphate Method.

Calcium phosphate-mediated transfection (Graham and van der Eb, 1973) involves mixing the DNA directly with $CaCl_2$ and phosphate buffer to form a fine calcium phosphate precipitate containing the DNA which is then placed on the cell monolayer. The precipitate binds to the plasma membrane and it is taken into the cell by endocytosis. In this new method Histone IIA (Sigma) was added to the $CaPO_4$ precipitate and mixed slowly and then spread on the plate of monolayer cells. Neuroblastoma cells were used due to their good transfection efficiency. A luciferase control plasmid (6μg) and CMV β-galactosidase plasmid (6μg) were used for the transfection and expression was quantified by the luciferase assay and a MUG β-galactosidase fluorescent assay.

Assay values obtained with the normal calcium phosphate method were considered as the control values and treated as the starting scale (1) to measure increase in the transfection efficiency (Table 1). There was no visible change in morphology of neuroblastoma cells. There was no transfection when histone alone was mixed with phosphate buffer or when DNA was mixed with calcium chloride alone. However when increasing amounts of histone (10μg/ml to 100μg/ml) were added after formation of the phosphate particles a 14 to 150 fold increase in β-galactosidase activity and 13 to 122 fold increase in luciferase activity was obtained. When 40μg/ml histone was added before or after formation of the precipitate then a 23-fold increase in β-galactosidase and a 45-fold or 74-fold increase in luciferase activity was obtained. Therefore it was observed that the addition of histone after formation of the calcium phosphate precipitate can increase transfection efficiency 120-150 fold, where the control was the traditional phosphate method.

Titration of the histone in the calcium precipitate was performed with lower amounts of the luciferase control plasmid (4μg) and 4μg of a Bluescript plasmid (Stratgene) (Table 8.2). Using increasing amounts of histone (10μg/ml to 100μg/ml), increases of 22 to 69 fold in N2A, 11 to 20 fold in 3T3 fibroblasts, 2-11 fold in C2 myoblasts and 2 fold in F9 EC cells were obtained.

Changes in morphology were observed in the F9 EC cells only, where cells formed circular colonies like embryoid bodies instead of a confluent monolayer of cells, resulting in decrease of cell number by almost 20 - 30 fold. However after removing the histone-calcium phosphate precipitate cells regained their original shape. There was no effect morphologically or transcriptionally on the D3 embryonic stem cells.

2. Histofection: Calcium Nitrate and histone Boost Transfection Efficiency.

After observing a substantial increase in the transfection efficiency with histone and calcium phosphate precipitate, it was found that calcium nitrate was useful for further increasing the transfection efficiency.

Calcium chloride was replaced with calcium nitrate for the formation of the calcium phosphate precipitate giving a 30-fold increase in transfection efficiency in N2A, 4-fold in 3T3 fibroblast and 2.4-fold in F9 EC cells. Subsequently, when histone was added to the calcium nitrate facilitated phosphate precipitate, the transfection efficiency was increased 305 to 405 fold in neuroblastoma cells (N2A), 15 to 16 fold in the fibroblasts (3T3) and 3-fold in the F9 EC cells. Calcium phosphate precipitate was also prepared from a commercially available Kit (FIVE PRIME TO THREE PRIME INC.) to act as a control for the precipitate formed. Values obtained from both sets of calcium chloride reagents were similar. When histone was added, similar increases in the transfection values i.e. 42 to 37 in N2A, 3 to 4 in 3T3 and 2 to 3 fold in F9 cells were obtained (Table 3).

Having achieved an increase in the transfection efficiency, the minimal amount of the luciferase control plasmid needed to achieve good transfection (Table 4) was assessed. With 1 ng of DNA, a 2-fold increase was obtained with the addition of histone. However with 500ng of DNA the increase with the histone was up to 9-fold. With 1μg of DNA a substantial increase of up to 18-64 fold was obtained.

Cells were stained for β-galactosidase activity in order to test whether the increase in the transfection efficiency was due to the DNA entering more cells, or whether there was more DNA going into each cell or an increased expression efficiency per cell was being observed. When cells were counted, a 6-8 fold increase was observed upon addition of

histone (Table 5). However, when the calcium chloride was replaced with calcium nitrate, a 5-fold increase was observed without histone addition, and upon histone addition a 22-33 fold increase in the cell number was obtained.

Other types of histones also increase transfection efficiency (Table 6). Classification of histones is based on the relative amounts of lysine and arginine. histone type IIA is moderately rich in lysine, whereas histone types III-SS and type V-S are members of the lysine rich subgroup.

H3A was superior with the calcium chloride method. With the nitrate method, H2A and H3A increased efficiency to 305 and 240 fold in N2A, 15 and 23 times in 3T3 and 3 and 6 times in F9 embryonal carcinoma cells. H5 was able to increase efficiency 2-14 fold by the chloride method and 2-194 fold by the nitrate method in various cell lines.

3. Histofection Increases G418-Clone Selection 4-Fold

A BAGLacZ, neo vector (12μg) was transfected in to ψCre producer cells. BAGLacZ, neo contains β-galactosidase as a marker gene and neomycin phosphotransferase as a selection gene. Transfections were done in duplicate with or without histone (80μg/ml) by the calcium chloride or nitrate method. After 48hr cells from each plate were split into 20 plates (10cm) with 10ml of DMEM medium containing 500μg/ml of G418 sulphate. Medium containing G418 sulphate was changed every 72 hrs. After three weeks G418 resistant clones were counted in duplicate sets of experiments.

With the control $CaCl_2$ method 740 clones were obtained; with addition of histone (80μg/ml) clones increased by 3-fold to 2120. However with the new method using $CaNO_3$ a 3.4-fold increase was observed where clones increased to 2540; with addition of histone (80μg/ml) clones increased slightly to 2820, thereby showing 4-fold increase in the transfection

efficiency.

These results demonstrate that there is an increase in transfection efficiency as a result of which an increase in the number of selected clones is observed.

TABLE 1. EFFECT OF HISTONE ON THE TRANSFECTION EFFICIENCY

REPORTER: pGL2 luciferase control plasmid (6µg).
pCMV β-galactosidase plasmid (6µg).
CELL LINE: Neuroblastoma cells (N2A)

METHOD*	β-galctosidase assay	luciferase assay
$CaPO_4$	1	1
+HIST 10µg/ml	14	13
+HIST 20µg/ml	24	23
+HIST 30µg/ml	91	41
+HIST 40µg/ml	85	74
+HIST 60µg/ml	100	63
+HIST 80µg/ml	130	122
+HIST 100µg/ml	150	77
+HIST 40µg/ml+	23	45
HIST 40µg/ml"	NIL	NIL
+DEAE Dextran 40µg/ml	0.3	1

*The $CaPO_4$ method (HBS buffer +DNA+$CaCl_2$ and histone type IIA (µg/ml of medium) were used.
+histone was added before addition of the $CaCl_2$.
"histone was added with the DNA only.

Values signify the fold increases compared to the standard calcium chloride method. 20µl of cell extract was analysed using the procedures and reagents supplied with the Luciferase Assay Reagent Kit (Promega). Luciferase activities were recorded by placing the reaction in a luminometer for 10 sec. These values were then divided by the protein concentration (in µg/µl) of the extract determined using the BIO-RAD protein assay kit with bovine serum albumin as standard. Such corrected values were used to calculate fold increases. β-galactosidase values were determined similarly using the Galactolight kit (TROPIX).

TABLE 2. EFFECT OF HISTONE ON THE TRANSFECTION EFFICIENCY ON DIFFERENT CELL LINES.

REPORTER: pGL2 luciferase control plasmid (4μg). pBluescript (4μg)
ASSAY: Luciferase assay

METHOD*	N2A	3T3	C2M	F9 EC**	D3 ES+	K562
$CaPO_4$	1	1	1	1	NIL	NIL
+HIST 10μg/m	122	11	2	2	NIL	NIL
+HIST 25μg/ml	26	12	3	1	NIL	NIL
+HIST 50μg/ml	36	20	11	1	NIL	NIL
+HIST 75μg/ml	54	8	5	1	NIL	NIL
+HIST 80μg/ml	69	5	4	1	NIL	NIL
+HIST 100μg/ml	28	14	1	1	NIL	NIL

*$CaPO_4$ method (HBS buffer + DNA + $CaCl_2$ and histone type IIA (concentration in μg/ml of medium) were used.
+D3 cells were stained for β-galactosidase activity which showed a few blue cells which were not sufficient for quantitation.
**F9 EC cells showed changes in the morphology and therefore the cell population decreased to a large extent at the initial stage.

Values signify the fold increases compared to the standard calcium chloride method. Analysis was performed as described in the legend to Table 1.
N2A, neuroblastoma 2A cells: 3T3, NIH3T3 fibroblasts: C2M, C2 myoblasts: F9EC, F9 embryonal carinoma cells: D3 ES, D3 embryonic stem cells: K562, K562 erythroleukaemia cells.

TABLE 3. HISTOFECTION: A NEW METHOD OF TRANSFECTION.

REPORTER: pGL2 luciferase control plasmid (4μg)
pBluescript (4μg)

ASSAY: Luciferase assay.

METHOD*	N2A	3T3	F9 EC
$CaCl_2$	1	1	1
+H40μg/ml	18	4	1.4
+H80μg/ml	42	3	1.4
$CaNO_3$	30	4	2.4
+H40μg/ml	402	26	2.0
+H80μg/ml	305	15	3.0
$CaCl_2$ (KIT)**	1	1	1.0
+H80μg/ml	37	4	3.0

*calcium chloride/nitrate were used to form the calcium phosphate precipitate and histone type II A was added in appropriate concentration (μg/ml of medium).

**The calcium phosphate kit was obtained from the FIVE PRIME TO THREE PRIME INC.

For details, see legends to Tables 1 and 2

TABLE 4. HISTOFECTION: EFFECT ON TRANSFECTION EFFICIENCY AS A FUNCTION OF THE AMOUNT OF DNA TRANSFECTED

REPORTER: pGL2 Luciferase control plasmid.
ASSAY: Luciferase assay.
CELL LINE: Neuroblastoma (N2A)

DNA (ng)	CALCIUM CHLORIDE - histone	CALCIUM CHLORIDE + histone	CALCIUM NITRATE - histone	CALCIUM NITRATE + histone*
1	7	12 (2)	6 (1.0)	12 (2.0)
50	17	65 (4)	60 (4.0)	145 (9.0)
100	60	147 (3)	85 (1.4)	140 (2.3)
250	201	605 (3)	226 (1.1)	960 (5.0)
500	234	1839 (8)	1099 (5.0)	4541 (2.5)
1000	233	3823 (18)	8822(38.0)	14846 (64.0)

The values in brackets show fold increase when compared to the standard calcium chloride (- histone) method.
* histone type IIA was used (80μg/ml of medium).

For details, see legend to Table 1

TABLE 5. HISTOFECTION: QUANTITATION OF THE TRANSFECTION EFFICIENCY BY COUNTING BLUE CELLS.

REPORTER: pCMV β-galactosidase plasmid (10μg).
ASSAY: β-galactosidase staining
CELL LINE: Neuroblastoma cells (N2A)

METHOD	MEAN COUNT* (FOLD INCREASE)
CALCIUM CHLORIDE	12
+ histone 40μg/ml	70 (6)
+ histone 80μg/ml	92 (8)
CALCIUM NITRATE	60 (5)
+ histone 40μg/ml	267 (22)
+ histone 80μg/ml	360 (33)

* Cells were counted at least six times at random sites on a 6 cm plate by using a 10x lens with a built in grid. Appropriate amount of histone type IIA was used with calcium chloride/ nitrate method.

β-galactosidase staining was performed by standard prcedures using 5-bromo-4-chloro-3-indoylyl-β-D-galactoside as the chromogeric substrate.

TABLE 6 HISTOFECTION: EFFECT OF DIFFERENT TYPES OF HISTONES ON THE TRANSFECTION EFFICIENCY.

REPORTER: pGL2 luciferase control plasmid (4μg)
pBluescript plasmid (4μg)
ASSAY: Luciferase assay.

histone TYPE	N2A	3T3	F9 EC
CALCIUM CHLORIDE METHOD			
H IIA	42	3	1.4
H IIIA	81	4	3.4
H IIA & IIIA*	63	9	1.2
H VA	14	2	1.2
CALCIUM NITRATE METHOD			
H IIA	305	15	3.0
H IIIA	240	23	6.0
H IIA & IIIA	281	7	4.0
H VA	194	6	1.4

histone concentration used in transfection was 80μg/ml of medium used. Values depicted in the table are the fold increases, when compared to the calcium chloride method (without histone).

* 40μg/ml of each type of histone was used for the transfection.

For details, see legends to Tables 1 and 2

CLAIMS:

1. A method for transforming a cell with a nucleic acid comprising contacting the cell with a vector which comprises the nucleic acid in the presence of a protein having a high basic amino acid content.

2. A method according to claim 1 wherein the nucleic acid is DNA.

3. A method according to claim 1 or claim 2 wherein the protein having a high basic amino acid content is a histone protein.

4. A method according to any preceding claim further comprising the steps of:

a) bringing the vector into admixture with calcium chloride in a phosphate buffer, to produce a calcium phosphate precipitate comprising the vector; and

b) contacting the cell with the calcium phosphate precipitate.

5. A method according to claim 4 wherein the protein having a high basic amino acid content is added after the formation of the calcium phosphate precipitate.

6. A method according to claim 4 or claim 5, wherein the calcium chloride is replaced by calcium nitrate.

7. A method for transfecting a cell with a nucleic acid comprising the steps of:

a) bringing the nucleic acid into the admixture with calcium nitrate in a phosphate buffer, to produce a calcium phosphate precipitate comprising the nucleic acid; and

b) contacting the cell with calcium phosphate precipitate.

8. A kit comprising at least one of:

a) a preparation containing a protein having a high basic amino acid content;

b) calcium chloride and/or calcium nitrate;

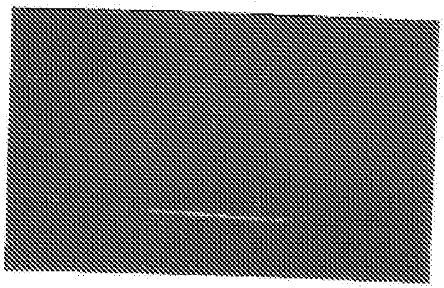
c) a phosphate buffer; and

d) nucleic acid.

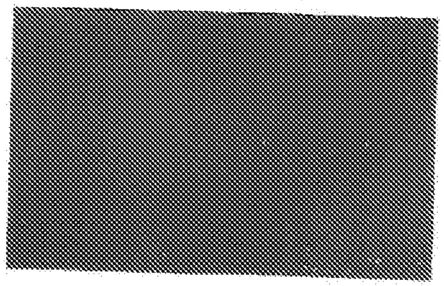
FIG.1

10 X MAG

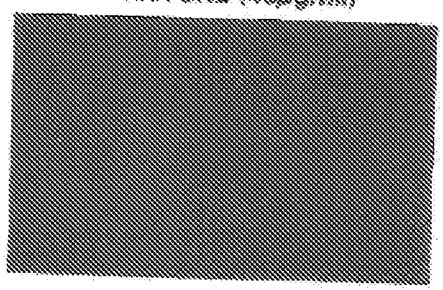
A. CaPO$_4$ METHOD



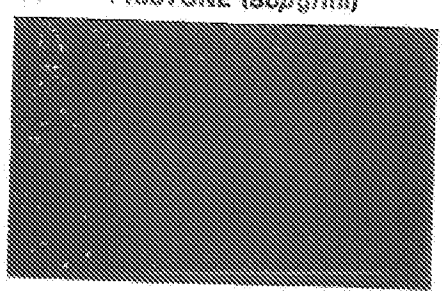
C. + HISTONE (20μg/ml)



D. + HISTONE (40μg/ml)

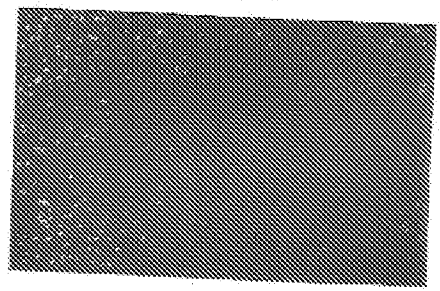


F. + HISTONE (80μg/ml)



20 X MAG

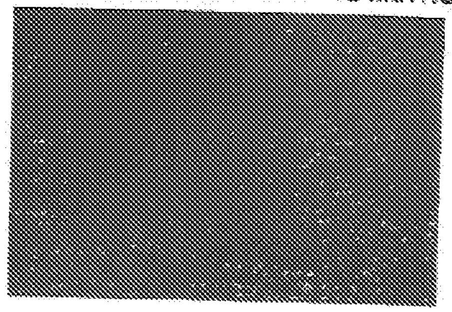
B. CaPO$_4$ METHOD



E. + HISTONE (40μg/ml)

G. + HISTONE (80μg/ml)

A. CALCIUM PHOSPHATE METHOD.



B. +HISTONE (20μg/ml).

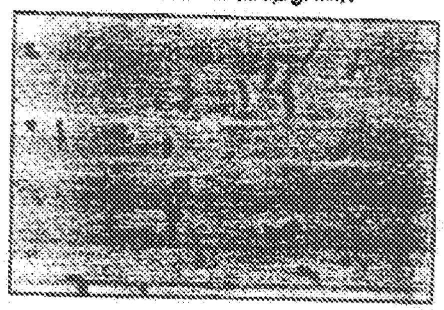
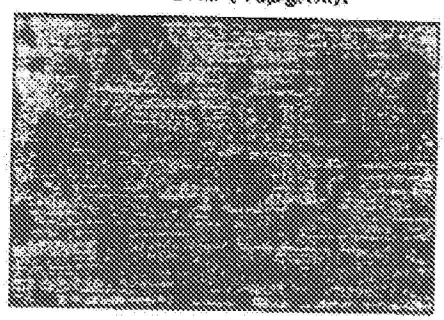


FIG.2

C. +HISTONE (40μg/ml).



D. +HISTONE (80μg/ml).

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/02612

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/87

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEMICA BIOPHYSICA ACTA, vol. 950, 1988 pages 221-228, M. BÖTTGER ET AL. 'Condensation of vector DNA by the chromosomal protein HMG1 results in efficient transfection' *see the whole article*	1-8
X	DD-A-256 148 (BÖTTGER M. ET AL.) 27 April 1988 *see the whole patent*	1-8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
30 January 1996	1 1. 03. 96

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 95/02612

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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X	ARCH. GEWULSTFORSCHUNG, vol. 60, no. 4, 1990 pages 265-270, M. BÖTTGER ET AL. 'Transfection of DNA-nuclear protein HMG1 complexes: raising efficiency and role of DNA topology' *see the whole article*	1-8
X	PLANT CELL REPORTS, vol. 12, 1993 pages 241-244, J.H. DOELLING ET AL. 'Transient expresssion in Arabidopsis thaliana protoplasts derived from rapidly established cell suspension cultures' *see the whole article*	6,7
X	DE-A-43 09 203 (C. HOLT) 21 April 1994 *see the whole patent*	1-4
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, vol. 88, 1991 pages 4255-4259, E. WAGNER ET AL. 'Transferrin-polycation-DNA complexes: the effect of polycations on the structure of the complex and DNA delivery to the cells' *see the whole article*	1-3
X	DE-A-41 10 409 (GENENTECH, INC.) 1 October 1992 *see the whole patent*	1-3
X	WO-A-94 25608 (BAYLOR COLEGE OF MEDICINE) 10 November 1994 *see the whole patent*	1-3
X	WO-A-91 17773 (BOEEHRINGER INGELHEIM INTERNATIONAL GMBH) 28 November 1991 *see the whole patent*	1-3
X	EP-A-0 388 758 (BOEHRINGER INGELHEIM INTERNATIONAL GMBH) 26 September 1990 *see the whole patent*	1-3

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Information on patent family members

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PCT/GB 95/02612

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		DE-D-	59106279	21-09-95
		EP-A-	0532525	24-03-93
EP-A-0388758	26-09-90	AU-B-	637085	20-05-93
		AU-B-	5137290	20-09-90
		CA-A-	2012311	16-09-90
		JP-A-	3200800	02-09-91
		US-A-	5354844	11-10-94